

PROTEIN AMINO ACIDS

Contents of Vegetable Leaf Proteins

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Leaf meals from beet, broccoli, carrot, celery, corn, kale, lima bean, pea, rhubarb, spinach, and turnip contain a nutritionally well-balanced mixture of histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, and tryptophan. The amino acid content of each leaf meal was found to be approximately 25% lower than that of the corresponding concentrate on a crude protein basis. That methionine values were as much as 73% lower was partly due to hydrolytic destruction.

IN VEGETABLE WASTE UTILIZATION studies carried on at this laboratory, many constituents of leaf meals prepared from the wastes have been investigated (19). Because the leaf meals are high in protein, knowledge of their amino acid contents is necessary for more efficient utilization of these products. Ten amino acids were determined—histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, and tryptophan.

Previous work by Chibnall (8), Lugg and Weller (13), Bailey (3), and others has shown that the determination of amino acids in leafy tissues is subject to error due to losses incurred when the proteins are hydrolyzed in acid in the presence of the carbohydrates normally in these materials. Armstrong (2) has recently reviewed this subject in connection with a study of the amino acid content of herbage. Dunn (9), who investigated this problem in connection with foods and food proteins, found indications of losses of methionine, threonine, lysine, phenylalanine, valine, and tryptophan in certain foods.

Before the general acceptance of microbiological procedures, the chemical methods of Block and Bolling (6) were used in determining amino acids in leaf meals. Failure to recover added amino acids showed that these methods were subject to such heavy losses that they could not be adapted for use with these materials.

Of the various microbiological methods available when this work was initiated, that of Stokes *et al.* (17) appealed because of its relative simplicity, in that it required only one set of reagents and

two bacterial organisms. In a recent cooperative study (7) this method was successfully used for the determination of amino acids in six selected protein foods.

Although formation of humin did not appear to be as great during the Stokes 10-hour autoclave hydrolysis as in the standard 24-hour reflux hydrolysis used with the chemical methods, there was, nevertheless, appreciable blackening in the tubes containing the hydrolyzed leaf meals. In view of this human formation, it was considered advisable to prepare leaf protein concentrates in which the amino acid content could be compared with that of the original leaf meal. Because the primary objective of this purification step was the elimination of as much carbohydrate as possible, methods of preparation of the leaf protein concentrates were chosen with this in mind.

Preparations

Leaf Meals Meals used in this study were prepared by the fractional drying procedure (19). They were dehydrated at approximately 240° F. in through-circulation laboratory or pilot plant dryers. For analysis, the dried products were ground to pass a 30-mesh screen and kept in storage at 40° F. in moistureproof containers.

Protoplasts The first method used for preparing a leaf protein concentrate was similar to that worked out by White *et al.* (20) at this laboratory. It involved the preparation of leaf protoplasts by anaerobic fermentation of the leaf tissue with the bacterium *Clostridium roseum*. This organism digests the cell walls, but the cell contents

(protoplasts) remain intact and can be separated as discrete entities from the bagasse (cuticle, ribs, vascular tissue) by mechanical screening. The protoplasts were recovered from the liquid phase by decantation, washing, and centrifuging. They were dried in a tray dryer with circulating air at 75° C. Protoplasts were prepared from fresh leaves of beet, broccoli, carrot, and lima bean.

Formic Acid Extracted Proteins The second method of preparing protein concentrates is similar to the method of Albanese (7). This procedure is based on the fact that proteins are soluble in warm formic acid, as noted by Mazur and Clarke (14), whereas fibrous material is not. Certain carbohydrates are soluble, however, and Albanese removed part of these by precipitation with alcohol.

One kilogram of fresh leaf blades was stripped from the petioles and heavier veins, frozen, covered with acetone, and extracted at room temperature for 48 hours. The drained leaves were bagged and extracted for 24 hours with acetone in a modified Soxhlet apparatus similar to that described by Albanese. The bagged leaves were dried in a current of warm air, after which the bag and contents were returned to the Soxhlet extraction chamber and enough 90% formic acid was added to cover the material. The extraction was allowed to proceed for 24 hours at 70° to 75° C. The first extract was discharged to the Soxhlet flask by addition of formic acid to siphon level, and a second extraction was carried out in a similar manner.

Table I. Amino Acids in Purified Proteins

(Calculated to 16.0% nitrogen)

	Histi- dine, %	Argi- nine, %	Lysine, %	Leucine, %	Iso- leucine, %	Valine, %	Methi- onine, %	Threo- nine, %	Phenyl- alanine, %	Trypto- phan, %
Albumin, egg	2.2	6.2	6.4	9.1	6.9	7.8	4.4	3.9	8.1	1.2
Stokes (77)	2.5	6.3	7.0	9.8	7.5	7.5	4.4	3.8	8.4	1.5
Block and Bolling (5) ^a	2.5	6.1	6.6	8.8	7.4	6.7	4.5	4.3	7.8	1.5
Rutgers (Stokes) (7)	2.4	5.4	6.6	8.5	6.5	7.2	3.8	5.0	6.5	1.6
Rutgers (best values) (7)	2.4	6.0	7.2	8.8	6.5	7.5	4.2	4.9	5.9	1.2
Tristram (78)	2.4	5.8	6.4	9.3	7.1	7.2	5.3	4.1	7.8	1.2
Albumin, bovine plasma	3.0	6.0	11.4	11.3	2.7	6.0	1.3	5.4	6.5	0.45
Block and Bolling (5) ^a	4.1	6.0	12.1	11.5	3.1	6.5	0.80	6.3	6.3	0.65
Stein and Moore (76)	4.0	5.9	12.8	12.2	2.6	5.9	0.81	5.8	6.6	0.58
Casein	3.1	4.0	7.7	9.9	6.0	7.3	2.8	4.3	6.1	1.2
Stokes (77)	3.0	4.2	8.2	10.6	6.0	7.1	2.8	4.5	6.3	1.2
Block and Bolling (5) ^a	3.1	4.0	8.2	10.5	6.9	7.2	2.9	4.2	5.3	1.3
Rutgers (Stokes) (7)	3.1	3.9	7.8	9.6	6.4	7.1	3.2	4.7	5.9	1.1
Rutgers (best values) (7)	3.0	3.9	8.1	10.0	6.4	7.4	3.2	4.5	5.4	0.96
Gordon (77)	3.2	4.2	8.4	9.4	6.2	7.3	2.9	5.0	5.1	1.2
β -Lactoglobulin	1.6	3.2	11.0	16.9	7.3	6.6	3.2	5.0	4.6	1.8
Stokes (77)	1.6	2.9	11.5	15.8	7.2	5.7	2.6	4.7	4.5	2.2
Block and Bolling (5) ^a	1.6	2.9	11.6	15.8	7.4	5.9	2.7		3.9	2.2
Tristram (78)	1.6	2.9	11.6	15.9	6.1	5.9	3.3	5.3	4.1	2.0

^a Averaged microbiological values.

The combined extracts were concentrated in vacuo to 1 liter and saved for further treatment. The extracted leaf material was stirred with 2 liters of 95% ethyl alcohol, and the strained and filtered alcohol-formic acid extract was adjusted to 2 liters with alcohol and added to the 1 liter of concentrated formic acid extract. The mixture was allowed to stand for 2 hours, and the precipitated carbohydrates were then filtered off.

The alcohol-formic acid filtrate was concentrated to a thick sirup, hydrochloric acid was added to give a 20% acid concentration, and the mixture was boiled under reflux for 24 hours. The hydrochloric acid was removed by three successive vacuum concentrations, with additions of water after each of the first two concentrations. The resulting sirup

was diluted to 300 ml. with water, and the humin precipitate was filtered out and extracted with boiling water. The filtrate plus the humin wash water was concentrated in vacuo to 200 ml. and used for amino acid analysis, basing the protein content on the total nitrogen determined by micro-Kjeldahl \times 6.25. This preparation is referred to as the formic acid extract.

Purified

The casein used in this investigation was a high-grade commercial sample having a protein content of 88.4%. Crystalline egg albumin, bovine plasma albumin, and β -lactoglobulin were obtained from G. W. Nutting and W. G. Gordon, both of this laboratory. The protein values were standardized for all experiments by determining micro-Kjeldahl nitrogen.

Methods of Analysis

To obtain more reproducible results, certain modifications of the Stokes procedure were necessary. The lactic acid production of *Streptococcus faecalis* R was increased by the addition of sodium citrate to the basal medium, as noted earlier by Baumgarten *et al.* (4). The medium was also sterilized as in Baumgarten's method by Seitz filtration, followed by inoculation with 2.5 ml. of the bacterial suspension. Five milliliters of the mixture was added, aseptically, to each tube of known amino acid solution or unknown hydrolyzate, which had previously been sterilized by autoclaving. The authors' results were also more consistent when an incubation time of 70 hours was used instead of the 40 hours recommended by Stokes.

Table II. Amino Acids in Leaf Meals

(Calculated to 16.0% nitrogen)

Source	Crude Protein Content, %	Histi- dine, %	Argi- nine, %	Lysine, %	Leucine, %	Iso- leucine, %	Valine, %	Methi- onine ^a , %	Threo- nine, %	Phenyl- alanine, %	Trypto- phan, %
Beet	24.3	1.3	4.1	5.4	6.4	4.2	5.1	1.7	3.8	5.8	1.2
Broccoli	41.0	1.5	4.8	4.5	6.4	3.2	4.5	1.8	3.3	6.0	1.4
Carrot	19.6	1.9	4.3	4.5	7.1	4.5	5.5	1.7	4.4	6.5	1.4
Celery	23.2	1.5	4.0	2.4	6.8	3.9	4.8	2.2	3.4	4.5	1.3
Corn	19.4	1.3	3.9	3.2	6.9	3.6	4.8	2.8	3.3	5.4	1.3
Kale	24.7	1.6	5.1	3.1	6.5	3.4	4.6	0.9	3.5	4.4	1.1
Lima bean	16.9	1.3	4.2	3.6	6.6	3.6	5.0	1.2	4.0	7.0	1.4
Pea	23.6	1.6	4.6	4.9	7.8	4.4	5.7	1.0	4.4	6.0	1.5
Rhubarb	26.1	1.9	4.7	5.4	8.4	4.0	5.3	1.0	4.0	6.1	1.6
Spinach	25.7	1.3	4.4	4.7	6.8	3.6	5.0	2.3	3.9	4.7	1.1
Turnip	23.9	1.4	4.5	3.0	6.8	3.9	4.8	2.2	4.0	5.3	1.3
Range	16.9-41.0	1.2-3.9	3.9-5.2	2.4-5.4	6.4-8.4	3.2-4.6	4.5-5.8	0.9-2.8	3.3-4.5	4.4-7.0	1.1-1.6
Other Leaf Meals from Literature ^b											
Alfalfa	18.1-19.4	1.2-2.1	3.1-4.3	3.6-4.9	6.2-6.6	3.6-5.2	4.1-4.4	0.2-1.4	2.2-3.6	4.1-4.6	1.0-1.4
Grass	19.4	3.1	6.7	7.2	13.4	9.3	10.3	2.1	6.7	8.8	2.1
Ryegrass	12.5	2.2	5.4	3.3	6.2	4.0	5.0	1.1	3.9	3.0	1.3

^a 2-hour hydrolysis.^b Block and Bolling (5).

With these modifications the results agreed within 10% or better.

A highly active strain of *Streptococcus faecalis* R, kindly supplied by C. M. Tuman of Texas A. and M. College, retained this activity for several years. A strain of *Lactobacillus delbrückii* LD 5 was kindly supplied by Merck & Co., Inc. Folic acid was obtained initially from R. J. Williams, University of Texas, and later as Folvite from B. W. Carey of Lederle Laboratories, to whom the authors are indebted.

Results and Discussion

To determine the reliability of the Stokes microbiological procedure, purified proteins were analyzed (Table I). Certain figures available in the literature (5, 7, 11, 16-18) for microbiological and chemical determinations are also summarized in this table. The greatest variation occurs in the determination of phenylalanine. The authors' values and those obtained by Stokes (17) and others using his procedure (7) are a little higher than the average of the microbiological values in the literature. With this exception, the authors' determinations agree well with average literature values.

Table II shows the amino acid contents of vegetable leaf meals. The values were obtained by 10 hours of acid or alkaline hydrolysis, with the exception of those for methionine.

Data for leaf protoplasts are given in Table III and for the formic acid protein extracts in Table IV.

Comparison of the amino acid contents of the leaf meals with those of their protein concentrates shows that the values for the meals when compared on the protein basis are appreciably lower than those for the concentrates. Table V was assembled from the data on three groups of the leaf preparations to show these differences. Eight of the amino

acids were from 13 to 26% lower in the leaf meals than in the averaged concentrates. Methionine after 10 hours of autoclave hydrolysis was 73% lower. Tryptophan was 33% lower in the leaf meals than in the protoplasts. The low value of methionine in the leaf meals indicated that it was undergoing destruction during the hydrolysis of these meals. A study of this loss (12) showed that a shorter period of autoclave hydrolysis gives a more complete recovery of this amino acid. The values listed in Table II are for 2 hours of autoclave hydrolysis, which has been shown by recovery tests to give better methionine values for the different meals than the 10-hour hydrolysis.

No comparable increase in the recovery of other amino acids from the leaf meal hydrolyzates was obtainable with shorter periods of hydrolysis, so it is doubtful if any of these were destroyed in this manner. When compared on the crude protein basis, the amino acid contents of the various leaf meals still averaged 24% lower than those in the concentrates. This indicated that the factor $N \times 6.25$ was not applicable to the estimation of crude protein in the leaf meals. The error in the method of calculating crude protein has long been recognized and adequately discussed by Block and Bolling (5), Ewing (10), and others. To correlate the amino acid contents of the leaf meals properly with those of the concentrates, it would be necessary to determine total nitrogen and amino acids in all the fractions resulting from the two methods of preparation of the concentrates. That a rough correlation exists is shown by the data on the formic acid extracts of eight leaf tissues. Twelve per cent of the total nitrogen was removed during the preliminary dehydration and fat extraction, and 13% remained in the residue after extraction

with formic acid. Although the form in which this 25% of the total leaf nitrogen occurs has not been determined, it is close to the 24% lower amino acid content of the leaf meals.

If one wishes to express the amino acid content of the vegetable leaf tissues on the crude protein basis, it would seem logical to use the values found in the protein concentrates (Tables III and IV) in preference to those found directly in the leaf meals (Table II).

From the viewpoint of the nutritionist, the amounts of the various amino acids per gram of meal are of greater importance than the amounts per gram of protein. The data on the leaf meals and on a number of commercial meals analyzed for comparison with the vegetable leaf meals are presented in Table VI.

The comparison shows that the vegetable meals are not concentrated sources of any of the amino acids, but they do have a well-balanced mixture, which would make them useful as supplementary sources of protein in animal or poultry diets. Solvent-extracted broccoli leaf meal was used by Runnels *et al.* (15) in high-energy poultry diets and gave excellent growth when combined with equal parts of soybean meal as the source of protein.

Summary

Vegetable leaf meals and protein concentrates prepared from the same leaf tissues were analyzed for histidine, arginine, lysine, leucine, isoleucine, valine, methionine, threonine, phenylalanine, and tryptophan by a modified Stokes microbiological procedure.

Leaf meals from beet, broccoli, carrot, celery, corn, kale, lima bean, pea, rhubarb, spinach, and turnip contain similar amounts of these ten amino acids, and although they are not outstandingly

Table III. Amino Acids in Leaf Protoplasts

(Calculated to 16.0% nitrogen)

Source	Crude Protein Content, %	Histidine, %	Arginine, %	Lysine, %	Leucine, %	Iso-leucine, %	Valine, %	Methionine, %	Threonine, %	Phenylalanine, %	Tryptophan, %
Beet	40.9	1.9	5.9	5.6	8.6	5.5	6.3	1.6	4.9	6.5	1.7
Broccoli, fat-free	78.2	1.8	5.2	5.3	8.9	4.7	5.9	1.8	4.4	7.8	2.3
Carrot	27.0	2.0	6.9	5.5	10.7	6.3	7.2	1.8	6.1	8.4	2.2
Lima bean	48.4	1.5	5.5	3.8	8.0	4.7	5.6	1.2	4.6	7.4	1.7

Table IV. Amino Acids in Leaf Proteins Extracted with Formic Acid

(Calculated to 16.0% nitrogen)

Source	Histidine, %	Arginine, %	Lysine, %	Leucine, %	Isoleucine, %	Valine, %	Methionine, %	Threonine, %	Phenylalanine, %
Broccoli	2.2	6.0	5.5	9.2	5.3	6.4	2.0	5.0	7.5
Carrot	2.1	5.7	5.4	10.6	5.5	7.0	3.4	5.0	7.7
Lima bean	1.9	5.7	5.2	9.1	5.2	5.9	2.2	4.7	7.0
Pea vine	1.8	5.4	5.8	8.7	5.1	6.1	1.8	4.5	6.7
Rhubarb	2.4	6.3	5.4	9.8	5.0	6.6	2.1	4.6	6.9
Rutabaga	1.7	5.4	5.6	7.2	4.7	6.0	1.6	4.2	5.8
Spinach	1.8	5.4	5.8	8.2	4.2	6.0	1.8	4.6	5.8

Table V. Comparison of Average Amino Acid Contents of Three Leaf Meals and Their Protein Concentrates
(Calculated to 16.0% nitrogen)

Leaf Preparation ^a	Histi- dine, %	Argi- nine, %	Lysine, %	Leucine, %	Iso- leucine, %	Valine, %	Methi- onine, %	Threo- nine, %	Phenyl- alanine, %	Trypto- phan, %
Meals	1.4	4.4	4.2	6.7	3.8	5.0	0.48	3.9	6.5	1.4
Protoplasts	1.8	5.9	4.9	9.2	5.2	6.2	1.6	5.0	7.9	2.1
Formic acid extract	2.1	5.8	5.4	9.6	5.3	6.4	2.5	4.9	7.4	...

^a Broccoli, carrot, lima bean.

high in any one acid they do contain a nutritionally well-balanced mixture.

The amino acid content of each leaf meal was approximately 25% lower than that of the corresponding concentrate when the comparison was made on the crude protein basis (micro-Kjeldahl nitrogen $\times 6.25$). Methionine values were as much as 73% lower, and part of the loss of this amino acid was found to be due to hydrolytic destruction. The lower values of all the amino acids were attributed to the failure of the $N \times 6.25$ factor to estimate protein correctly in the leaf meals.

Acknowledgment

The authors wish to express their sincere thanks for the help of two of their coworkers, who contributed greatly to the early phases of this work. Raymond Merrow prepared most of the formic acid protein extracts and Ruth Sutton initiated and modified the Stokes procedure. The authors thank J. J. Willaman in particular for his helpful suggestions and his careful review of the manuscript.

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Table VI. Amino Acid Contents of Leaf Meals and Protein Feeds
(Calculated to dry basis)

Source	Crude Protein Content, %	Histi- dine, %	Argi- nine, %	Lysine, %	Leucine, %	Iso- leucine, %	Valine, %	Methi- onine ^a , %	Threo- nine, %	Phenyl- alanine, %	Trypto- phan, %
Leaf Meals											
Beet	26.0	0.34	1.1	1.4	1.7	1.1	1.3	0.44	1.0	1.5	0.31
Broccoli	43.9	0.66	2.1	2.0	2.8	1.4	2.0	0.79	1.5	2.6	0.61
Carrot	21.1	0.25	0.91	0.95	1.5	1.0	1.2	0.36	0.93	1.4	0.30
Celery	26.0	0.39	1.0	0.62	1.8	1.0	1.3	0.57	0.88	1.2	0.34
Corn	21.9	0.29	0.86	0.70	1.5	0.79	1.1	0.62	0.73	1.2	0.29
Kale	24.9	0.40	1.3	0.77	1.6	0.85	1.2	0.22	0.90	1.1	0.27
Lima bean	17.8	0.23	0.75	0.64	1.2	0.64	0.89	0.21	0.71	1.3	0.25
Pea	25.1	0.40	1.2	1.2	2.0	1.1	1.4	0.25	1.1	1.5	0.38
Rhubarb	28.4	0.54	1.3	1.5	2.4	1.1	1.5	0.28	1.1	1.7	0.45
Spinach	27.4	0.36	1.2	1.3	1.9	1.0	1.4	0.63	1.1	1.3	0.30
Turnip	26.1	0.37	1.2	0.78	1.8	1.0	1.3	0.58	1.1	1.4	0.34
Av.	26.2	0.38	1.2	1.1	1.8	1.0	1.3	0.45	1.0	1.5	0.35
Protein Feed Meals											
Corn gluten	60.0	1.1	1.9	0.66	9.4	2.7	2.8	1.1	2.0	4.0	0.29
Crab	35.0	0.53	2.0	1.6	2.0	1.3	1.7	0.63	1.2	1.7	0.39
Fish	69.2	1.5	4.7	4.1	4.8	3.3	3.8	1.7	3.1	3.1	0.70
Meat and bone scrap	54.5	0.82	4.1	2.0	3.1	1.5	2.2	0.65	1.6	1.8	0.26
Soybean	48.8	1.1	3.4	1.8	3.8	2.4	2.6	0.49	1.9	2.5	0.54

^a 2-hour hydrolysis.